EXERCISE 15

Capsule Stain

After doing this exercise, you should be able to:

1. Perform a capsule stain on bacterial smears.
2. Describe the structure and function of a bacterial capsule.
3. Explain how the capsule stain works.
4. Understand the clinical significance of the bacterial capsule.
5. Perform oil-immersion microscopy to view your own capsule stained bacterial samples.

Background

The capsule stain is a structural stain that allows us to view bacterial **capsules**, a sticky extracellular polysaccharide or peptide layer that certain bacteria rely on for adhesion and protection. Because bacterial capsules have a neutral charge, they do not readily take up acidic or basic dyes. The capsule stain technique takes advantage of this characteristic by staining *around* the cells with an acidic stain such as Congo red or nigrosin and then staining the cell itself with a basic dye. Provided the bacterial species you stained makes a capsule, the final effect is quite pretty; you'll see a colored slide background dotted with stained cells surrounded by a white halo (the unstained capsule) (Fig. 15.1).

The capsule stain incorporates some aspects of negative staining, namely we stain the background of the slide and we do *not* heat fix the sample. Avoiding heat fixation is essential because applying heat will cause cell shrinkage, which can leave an artifactual white halo around cells that might be interpreted as a capsule. Plus, capsules are fragile, so heating the preparation can distort the structure. In place of heat fixing, cells are emulsified in a drop of serum to promote their adhering to the glass slide. The serum can also enhance the size of the capsule so that it is easier to visualize.

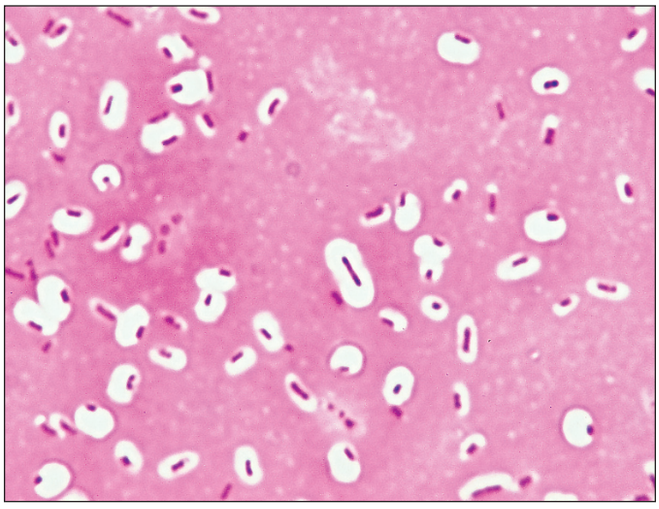


FIGURE **15.1** **Capsule Stain** • The acidic stain colorizes the background while the basic stain colorizes the cell, leaving the capsules as unstained, white clearings around the cells. Notice the lack of uniform capsule size, and even the absence of a capsule in some cells. There are several variations of the capsule stain. The capsule stain you will perform in this exercise produces different colors than shown in this micrograph, but the principle is the same, capsules appear as white clearings around cells.

Clinical Applications

A number of important Gram-positive and Gram-negative bacterial pathogens make capsules, examples include: *Bacillus anthracis* (anthrax), *Streptococcus pneumoniae* (bacterial pneumonia), *Haemophilus influenzae* (bacteremia, epiglottitis, pneumonia, and meningitis), *Neisseria meningitidis* (meningococcal meningitis), *Klebsiella pneumoniae* (pneumonia), *Salmonella typhi* (typhoid fever), and others. For these bacteria, having a capsule is an important **virulence factor** (a feature that helps an infectious agent cause disease). Not only does having a capsule help a pathogen adhere to surfaces, this extracellular structure also renders bacteria less vulnerable to phagocytosis, an important process that certain white blood cells use to clear invaders from the body. The capsule stain allows us to detect cells capable of producing an extracellular capsule and can therefore reveal information about the bacterium in question.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Clean glass microscope slides
- ❑ Squirt bottle of distilled water
- ❑ Bibulous paper and/or paper towels
- ❑ Staining tray or glass beaker to collect dye waste rinsed off slides
- ❑ Slide holder
- ❑ Sterile toothpicks
- ❑ Sheep serum (optional)
- ❑ Maneval's stain
- ❑ Congo red stain
- ❑ Coplin staining jar (or a beaker) with distilled water
- ❑ Recommended organisms (18- to 24-hour skim milk or tryptic soy agar [TSA] slant pure cultures):
  - ❑ *Aeromonas sobria*
  - ❑ *Rhizobium leguminosarum*
  - ❑ *Klebsiella pneumoniae* (BSL-2) (optional)

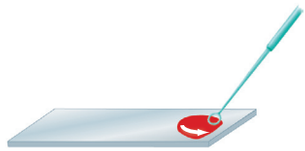
PROCEDURE

The capsule stain allows you to visualize an extracellular capsule, if present. Be careful to distinguish between a tiny white halo (a result of cell shrinkage) and a true capsule. While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Stain the Bacteria:** Follow the procedure illustrated in Figure 15.2 to stain your bacteria. Use a separate slide for each specimen. ***Note:*** Unlike our prior procedures, in this procedure we do not prepare heat-fixed bacterial smears. We do not apply heat at any point in the procedure. Instead we place the bacteria into a drop of sheep serum and dye on the slide and drag it across the slide with a second slide. This procedure is similar to the negative stain procedure in Exercise 12. ***Caution!*** Use BSL-2 precautions if *K. pneumoniae* is stained.
2. **Prepare a Gumline Sample:** Remove your gloves and wash your hands. Then, using a sterile toothpick, obtain a sample from below the gumline in your mouth. (Do not draw blood!) Mix the sample into a drop of water or serum on a slide, and then perform a capsule stain on it.
3. **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of the bacterium's cell shape, arrangement, and capsule features (if present). Record your observations in the data sheet at the end of this lab.
4. **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

1. Add Dye to the Slide

Place the slide on a paper towel on the bench space. Place a drop of sheep serum at one end of a clean slide. Then, add a drop or loopful of Congo red stain. Be sure to wear gloves.

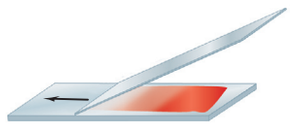


2. Add Bacteria to the Slide

With the slide resting on the paper towel-covered bench space, use an inoculating loop to transfer a small amount of bacteria into the serum-dye mixture. Don't over-inoculate and avoid spattering the mixture. Sterilize the loop after emulsifying. ***Note:*** If transferring a BSL-2 organism, use a sterile wooden stick or disposable loop/needle and dispose of it properly when finished.

3. Angle a Spreader to Contact the Emulsion

Here we use a second clean slide (or a glass coverslip) as a "spreader." Place the spreader at a roughly 30° angle so it contacts the emulsion.



4. Drag the Emulsion Across the Slide

Using a gentle and smooth motion, drag the spreader across the slide so the emulsion is thinly spread over the slide. This should be one uninterrupted and fluid motion; do not pick up the spreader until the end of the slide is reached or you will have a choppy preparation. Dispose of the spreader slide in a jar of disinfectant or sharps container.

5. Air-Dry the Slide

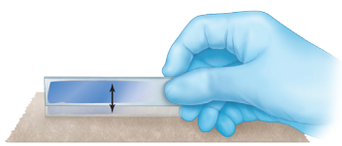
Allow the sample to air-dry, do not heat it at all.

6. Add the Second Dye

Place the slide over a staining tray or beaker and cover the spread with Maneval's stain for 1 minute.

7. Rinse Off the Maneval's Stain

Grasp the slide with a slide holder and dip it into a jar of distilled water two or three times to rinse off the Maneval's stain. Change the water in the jar frequently.



8. Remove Excess Water

Gently tap the edge of the slide on a paper towel to remove excess water, then set it down to air-dry. When dry, observe under oil immersion.

FIGURE **15.2** **Capsule Stain Procedure**

— Lab Notes —

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 15

DATA SHEET

Capsule Stain

During the Lab

1 Record your observations in the provided table. Use a separate line for each organism from your gumline sample that illustrates a capsule or an unusual cell morphology (cell shape and/or arrangement).

Organism	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells)	Capsule (+/–)

Lab Related Questions

1 How many dyes are used in the capsule stain?

a. 0
b. 1
c. 2
d. 3
e. 4

2 Choose the true statement(s) about capsules (select all that apply).

a. Capsules appear colorless after a properly performed capsule stain.
b. All bacteria make capsules.
c. Capsules are a virulence factor for some bacteria.
d. Capsules are made of polysaccharides.
e. Capsules are only made by select Gram-positive bacteria.

3 The capsule stain is considered a(n) _____ stain.

a. selective
b. structural
c. basic
d. acidic
e. simple

4 Some of the following steps are used in the capsule staining procedure, but some of the steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Decolorize the slide.

_____ Add Maneval's stain to the slide.

_____ Add Congo red to the slide.

_____ Add carbolfuchsin to the slide.

_____ Heat fix the samples on the slide.

_____ Add bacteria to the slide.

5 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ The capsule stain requires steaming the dye into the capsule.

_____ The capsule staining technique incorporates aspects of the negative staining technique.

_____ The capsule stain involves an acidic dye and a mordant.

_____ Bacteria that have a capsule are resistant to phagocytosis.

6 Capsules are a(n) _____ structure.

a. intracellular
b. extracellular
c. cell wall
d. cellulose
e. heat-resistant

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 15
DATA SHEET
(continued)

Critical Thinking

1 Some oral bacteria produce an extracellular capsule. Why may a capsule benefit these cells?

2 If having a capsule is an important advantage for certain pathogens, then why wouldn't evolution favor all pathogens being encapsulated?

— Lab Notes —

EXERCISE 16

Endospore Stain

After doing this exercise, you should be able to:

1. Perform an endospore stain on bacterial smears.
2. Describe the structure and function of bacterial endospores.
3. Explain how the endospore stain works.
4. Understand the clinical significance of bacterial endospores.
5. Perform oil-immersion microscopy to view your own endospore stained bacterial samples.

Background

In this lab exercise, you will perform a structural stain called the **Schaeffer-Fulton method** to stain **endospores**, dormant structures that some bacterial species can make in response to harsh environmental conditions (e.g., nutrient depletion, unfavorable temperatures [from boiling to freezing], radiation, high population density, etc.). Because endospores are highly resistant to heat and chemicals (including disinfectants and drugs), the capacity to convert into this dormant form (undergo **sporulation**) allows bacteria to survive adverse conditions for long periods. The record for longevity goes to bacterial spores that were trapped in salt crystals about 250 million years ago and revived in a lab in the year 2000.

Endospores' resistance is due to a combination of factors, including a tough outer covering made of the protein **keratin**, its dehydrated state; DNA protective proteins; and other adaptations. When conditions are suitable again, endospores germinate into metabolically active **vegetative cells**. The keratin in the spore coat also resists staining; therefore, in the Schaeffer-Fulton method (Fig. 16.1), we use steam to drive the primary stain, malachite green, into spores. Alternatively, malachite green can be left on the slide for 15 minutes or more to stain the spores. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells and **spore mother cells** (which are responsible for producing the endospore) are easily decolorized with water and then counterstained with safranin (Fig. 16.2).

Endospores may be in the middle of the cell (**central**), at the end of the cell (**terminal**), or between the end and middle of the cell (**subterminal**). In some species, endospore location is variable, cells may exhibit a combination of terminal and subterminal, for instance. We also classify endospores by shape—either **spherical** or **elliptical** (**oval**)—and size relative to the cell (that is, whether they cause the mother cell to look swollen or not). Figures 16.3 through 16.5 show the diversity of endospore features.

The genera *Clostridioides*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Sporolactobacillus*, *Sporosarcina*, *Thermoactinomyces*, and *Oscillospira* are all examples of endospore-forming bacteria. Among these, *Bacillus*, *Clostridium*, and *Clostridioides* are the clinically important groups. In this exercise, we focus on *Bacillus* as our endospore model. Given enough time, *Bacillus* species will start to make endospores in most media that support their growth. However, we can accelerate sporulation by enriching growth media with manganese because several sporulation enzymes require manganese as a required cofactor. Your instructor may supply you either with cultures grown in sporulating agar or with older cultures not grown on such agar.

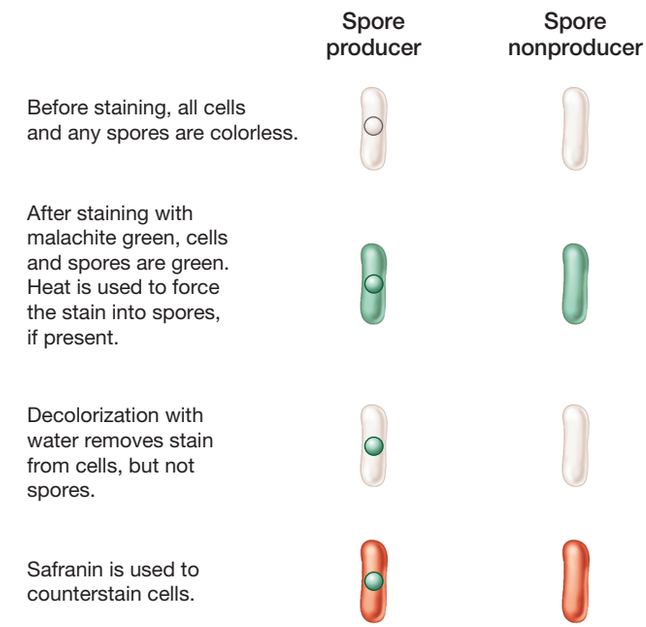

Spore producer
Spore nonproducer
Before staining, all cells and any spores are colorless.
After staining with malachite green, cells and spores are green. Heat is used to force the stain into spores, if present.
Decolorization with water removes stain from cells, but not spores.
Safranin is used to counterstain cells.

FIGURE **16.1** **Schaeffer-Fulton Spore Stain Overview** • Upon completion, endospores are green, vegetative and spore mother cells are red.

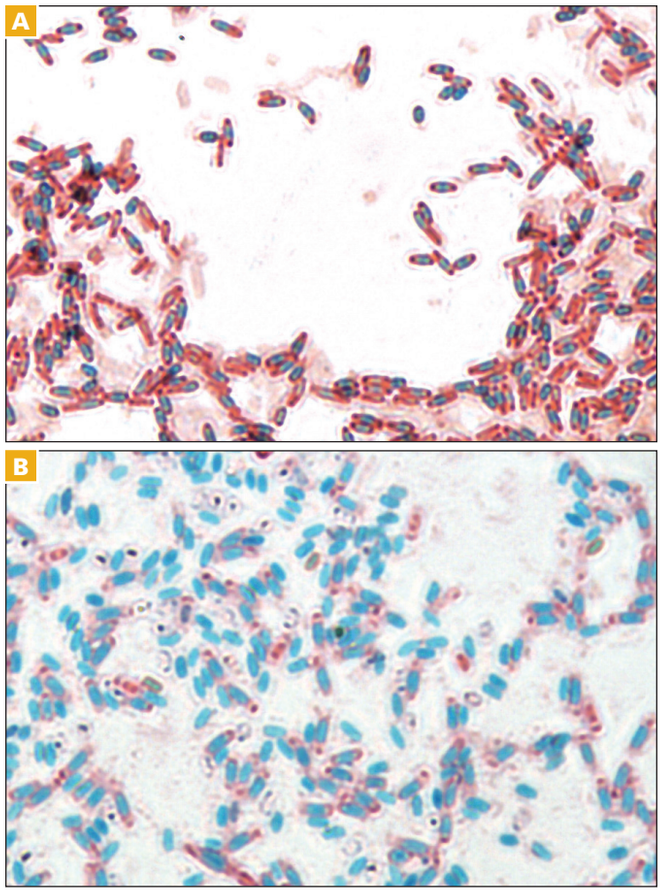


FIGURE **16.2** **Culture Age Can Affect Sporulation** • Bacteria that can form endospores do so in response to environmental stress, so as nutrients are depleted in older cultures, sporulation is more common. These two cultures illustrate different degrees of sporulation. (A) Most cells in this specimen contain endospores; very few have been released. The light pink rods are probably spore mother cells that have released their spore and have died. (B) This specimen consists mostly of released endospores.

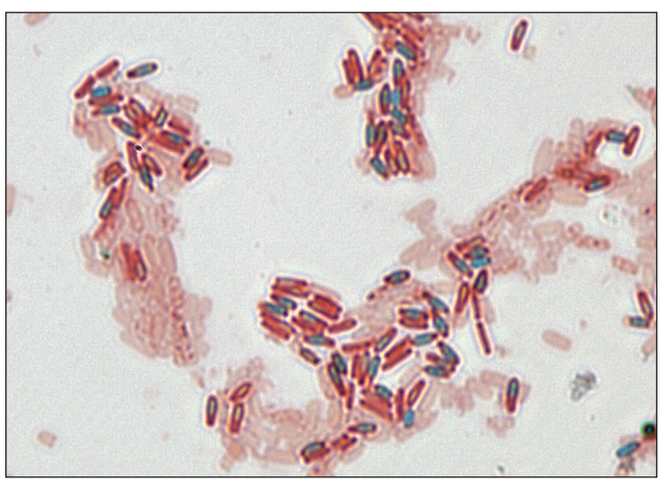


FIGURE **16.3** **Central Elliptical Endospores** • Most of these *Bacillus megaterium* endospores are centrally positioned, but some are subterminal. All are elliptical, but they do not distend the mother cell.

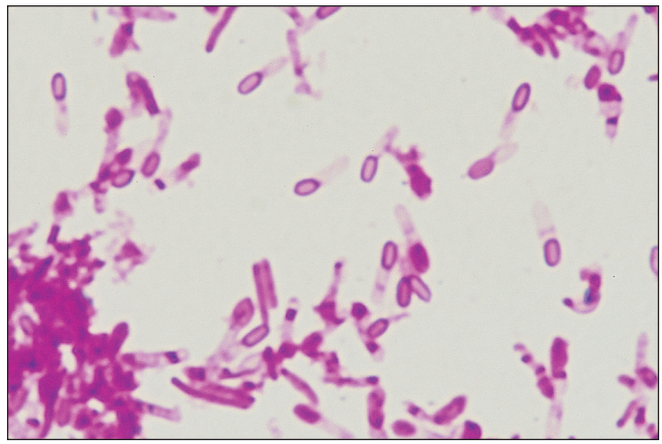


FIGURE **16.4** **Terminal Elliptical Endospores** • Shown is *Clostridium tetani* stained by a different endospore stain protocol using carbolfuchsin. Notice how the endospores have caused the ends of the cells to distend (swell).

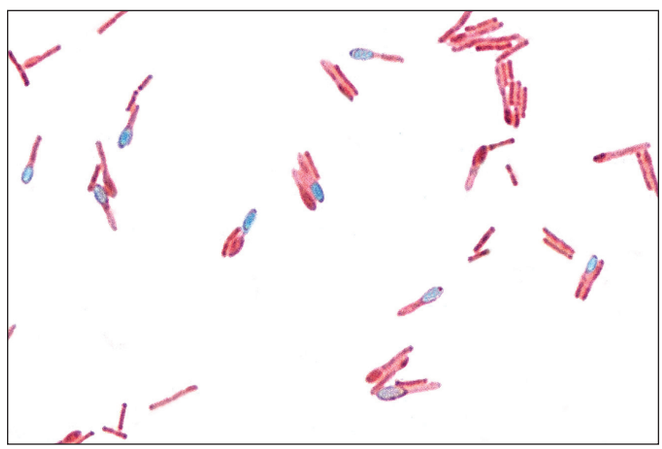


FIGURE **16.5** **Subterminal Elliptical Endospores** • In these *Clostridium butyricum* cells, most of the green endospores are "capped" by red cytosol, indicating that they are subterminal. Also notice how the spores distend the mother cell, an easily identified characteristic.

A Note on Endospore Function

Students often confuse bacterial spores with fungal spores. It's important to know that unlike fungal spores, bacterial spores do *not* have a reproductive function. A key goal of reproduction is to increase a population. Although the terminology "spore mother cell" may imply a reproductive capacity, endospore formation is not reproductive. Instead, it simply allows the original cell to enter a dormant spore state to weather out harsh conditions and germinate when the environment is more hospitable. The phrase "spore mother cell" is used to emphasize that the cell gives rise to a given spore. Upon spore completion, the mother cell (or rather, what remains of the original cell) is simply a husk that, once destroyed, releases the spore into the environment.

Clinical Applications

The endospore stain is a differential stain used to detect the presence, shape, and location of endospores in bacterial cells. Only a few genera produce spores. Most common are the genera *Bacillus* (more than 100 species) and *Clostridium* (more than 160 species). In addition, several "new" genera formerly classified as *Bacillus* species are also endospore producers. These include *Brevibacillus*, *Geobacillus*, *Paenibacillus*, and *Virgibacillus*, among others.

Most *Bacillus* species are soil, freshwater, or marine **saprophytes** (organisms that live off decaying organic matter), but two are pathogens. *B. anthracis* causes anthrax, and *B. cereus* causes two kinds of food poisoning: emetic and diarrheal. Most *Clostridium* members are harmless soil or aquatic saprophytes; some are notorious pathogens—namely, *C. tetani* (tetanus), *C. botulinum* (botulism), and *C. perfringens* (gas gangrene).

Because endospores are especially resistant to chemicals, heat, and other environmental stresses, they are quite challenging to destroy both in patients and in clinical environments. For example, the pathogen *Clostridioides difficile* (formerly *Clostridium difficile*), which causes infectious diarrhea, can enter the dormant endospore state in response to drug therapies. Thus, it is common for patients who successfully recover from a bout of *C. difficile* diarrhea to relapse within weeks as the spores germinate and reenter an actively growing vegetative state.

MATERIALS

- ❑ Lab coat
- ❑ Disposable gloves
- ❑ Safety glasses
- ❑ Compound microscope with oil-immersion lens
- ❑ Immersion oil
- ❑ Lens paper
- ❑ Clean glass microscope slides
- ❑ Squirt bottle of distilled water
- ❑ Bibulous paper and/or paper towels
- ❑ Staining tray or glass beaker to collect dye waste rinsed off slides
- ❑ Heating apparatus (beaker of water on a hot plate)
- ❑ Slide holder
- ❑ Malachite green stain
- ❑ Safranin stain
- ❑ Recommended organisms:
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus cereus*
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus coagulans*
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus megaterium*
  - ☐ 24-hour and 48-hour sporulating agar slant pure cultures of *Bacillus subtilis*

Note: Tryptic soy agar can be substituted for sporulating agar, but incubation times should be adjusted to 48 hours and 5 days.

Recipe for Sporulating Agar

Pancreatic digest of gelatin	6.0 g
Pancreatic digest of casein ($C_{38}H_{57}N_9O_9$)	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose (glucose) ($C_6H_{12}O_6$)	1.0 g
Agar	15.0 g
Manganous sulfate ($MnSO_4$)	0.3 g
Distilled or deionized water	1.0 L

Final pH 6.6 ± 0.2 at 25°C

PROCEDURE

Today you will visualize endospores of as many as four *Bacillus* species. To do this, you will use the Schaeffer-Fulton endospore staining method. In addition, you will be supplied with two cultures of different ages of each species to see if there is a relationship between culture age and endospore production. While performing the staining procedure, wear a lab coat, gloves, and safety glasses. You may remove your gloves and eye protection when using the microscope.

1. **Prepare the Bacterial Smears:** For each organism, prepare and heat fix two smears on the same slide. One smear should be the 24-hour culture, and the other the 48-hour culture (or 48-hour and 5-day cultures if TSA is the medium). Divide the work of slide preparation within your lab group. Minimally, each student should prepare one slide with smears of the 24-hour and 48-hour cultures of one species. ***Caution!*** Use BSL-2 precautions if you have subcultured soil isolates for endospore staining.

2 **Stain the Bacteria:** Follow the procedure illustrated in Figure 16.6 to stain your bacteria. Use a steaming apparatus like the one shown in Figure 16.7. Perform the steaming step in a well-ventilated area (preferably inside a fume hood). Always avoid inhaling the fumes. If you must carry the slide to and from the steaming apparatus, put it in a covered petri plate.

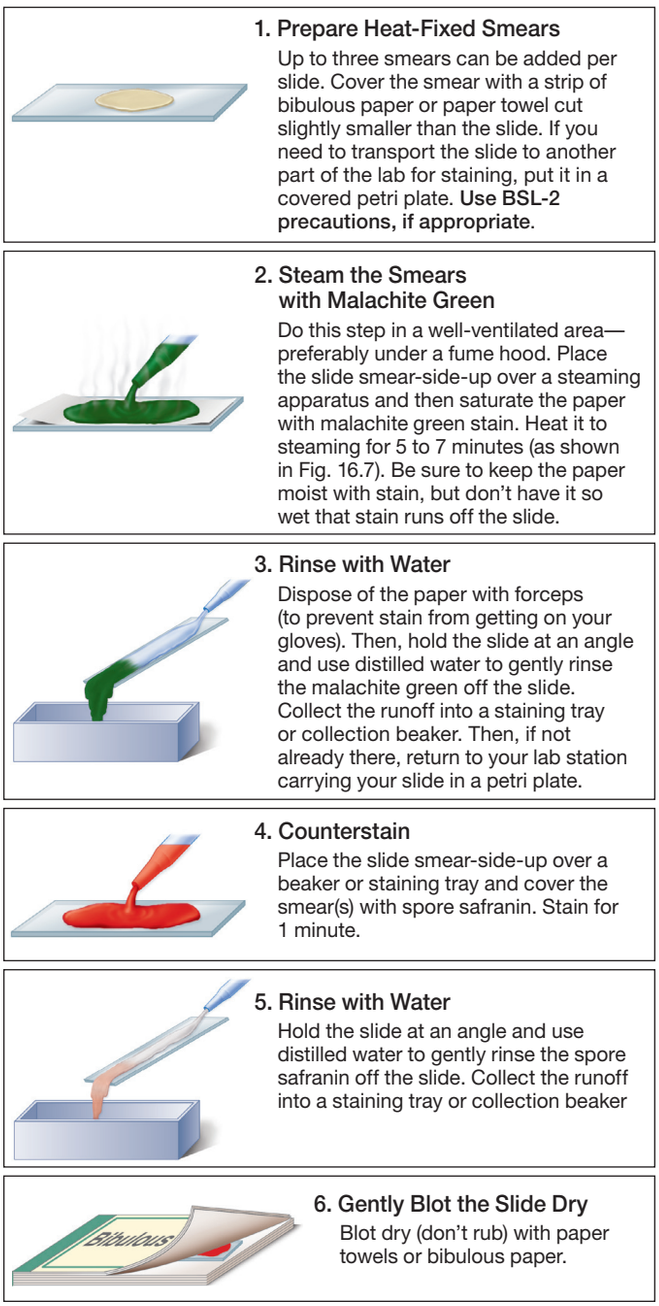

1. Prepare Heat-Fixed Smears
Up to three smears can be added per slide. Cover the smear with a strip of bibulous paper or paper towel cut slightly smaller than the slide. If you need to transport the slide to another part of the lab for staining, put it in a covered petri plate. Use BSL-2 precautions, if appropriate.
2. Steam the Smears with Malachite Green
Do this step in a well-ventilated area—preferably under a fume hood. Place the slide smear-side-up over a steaming apparatus and then saturate the paper with malachite green stain. Heat it to steaming for 5 to 7 minutes (as shown in Fig. 16.7). Be sure to keep the paper moist with stain, but don't have it so wet that stain runs off the slide.
3. Rinse with Water
Dispose of the paper with forceps (to prevent stain from getting on your gloves). Then, hold the slide at an angle and use distilled water to gently rinse the malachite green off the slide. Collect the runoff into a staining tray or collection beaker. Then, if not already there, return to your lab station carrying your slide in a petri plate.
4. Counterstain
Place the slide smear-side-up over a beaker or staining tray and cover the smear(s) with spore safranin. Stain for 1 minute.
5. Rinse with Water
Hold the slide at an angle and use distilled water to gently rinse the spore safranin off the slide. Collect the runoff into a staining tray or collection beaker
Bibulous
6. Gently Blot the Slide Dry
Blot dry (don't rub) with paper towels or bibulous paper.

FIGURE **16.6** **Schaeffer-Fulton Endospore Staining Procedure** • Perform this procedure with adequate ventilation (preferably in a fume hood), eye protection, a lab coat, and gloves. ***Caution!*** Employ BSL-2 precautions if you have subcultured a soil isolate for endospore staining.

3 **Observe Your Slides:** Use a light microscope to observe each slide via bright-field microscopy. If you need a refresher on how to get a sample in focus under oil immersion, refer to Exercise 1. Once the specimen is in focus under the oil-immersion lens, make your observations of visible endospores for each species. Be sure to note their shapes, locations, and relative sizes.

4 **Dispose of Your Slides:** Dispose of the slides and used stain according to your laboratory's policy. Used slides are typically discarded into a biological sharps container and stain waste is collected into a container for proper chemical removal.

FIGURE **16.7** **Steaming the Slide During the Endospore Stain** • Carefully steam the slide to force the malachite green into the endospores. Do not boil the slide or let it dry out. Keep it moist with stain for up to 7 minutes of steaming. ***Caution!*** This should be performed in a well-ventilated area (preferably in a fume hood) while wearing a lab coat, safety glasses, and gloves.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 16

DATA SHEET

Endospore Stain

During the Lab

1 Record your observations in the provided table. Use a separate line for each bacterial smear you observed.

Organism (include culture age)	Cellular Shape and Arrangement (morphology) (include a written description and a detailed sketch of some representative cells and spores)	Spores (present or absent)	Spore Shape and Position (if present)

Lab Related Questions

1 What is the primary stain in the Schaeffer-Fulton endospore staining method?

a. Methylene blue
b. Malachite green
c. Safranin
d. Crystal violet
e. Carbolfuchsin

2 Choose the true statement(s) about endospores (select all that apply).

a. Endospores resist staining.
b. Endospores stain pink in the endospore staining procedure.
c. *Bacillus* species make endospores.
d. Endospores are dormant structures.
e. Endospores are heat sensitive.

3 Mark each statement as true or false **and** correct the false statements so they read as true.

_____ An endospore's heat resistance is in part due to its low water content.

_____ Endospores serve as reproductive structures.

_____ Endospores are a clinical challenge because they can resist drug treatments.

_____ Adding magnesium to growth media can encourage spore-forming bacteria to make endospores.

4 The Schaeffer-Fulton endospore staining method is considered a(n) _____ stain.

a. acidic
b. basic
c. selective
d. simple
e. structural

5 Some of the following steps are used in the Schaeffer-Fulton endospore staining method, but some of the steps are not used. First, draw a line through the steps you would *not* use. Next, place the remaining steps in sequential order, with "1" being the earliest step.

_____ Rinse the primary stain off the slide with water.

_____ Steam the slide with the primary dye for 5–7 minutes.

_____ Decolorize the slide with acid-alcohol.

_____ Add safranin dye to the slide.

_____ Add Maneval's stain to the slide.

_____ Heat fix the samples on the slide.

_____ Rinse the counterstain off the slide with water.

_____ Blot the slide dry.

Name _____ Date _____

Lab Section _____ I was present and performed this exercise (initials) _____

EXERCISE 16
DATA SHEET
(continued)

6 The process of endospore formation is called _____.

7 Endospores will appear _____ while mother cells appear _____ at the end of the Schaeffer-Fulton endospore staining method.

a. green; blue
b. blue; green
c. green; red
d. red; green
e. green; violet

Critical Thinking

1 Design an endospore staining procedure where carbolfuchsin and methylene blue are used as the dyes. Describe your protocol based on the fundamentals of the Schaeffer-Fulton endospore staining method.

2 Some people harbor *Clostridioides difficile* as a natural inhabitant of their gastrointestinal tract. However, this bacterium is unlikely to cause diarrhea unless the person recently underwent an antibiotic regimen (especially a broad-spectrum antibiotic treatment). Why would taking a broad-spectrum antibiotic treatment put a patient at risk for *C. difficile* diarrhea?

— Lab Notes —